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TITLE: Lowering T Cell Activation Thresholds and Deregulating Homeostasis to Facilitate Immunotherapeutic Responses to Treat Prostate Cancer

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## ABSTRACT.

The induction of tumor-specific T cells remains a primary obstacle to immunotherapeutic approaches for most cancers including prostate cancer. This difficulty has been largely ascribed to mechanisms for tumor evasion of the immune system and host-imposed restrictions (collectively referred to as tolerance) that prevent cross-reactive autoimmunity against the parent tissues from which tumors arise. Limitations in techniques to identify novel and truly immunogenic prostate-specific antigens and efficient methods to modify autologous tissues for vaccine preparation have further constrained approaches to develop immune-based therapies for prostate cancer. Hence, relatively straightforward manipulations that induce specific T cell responses against prostate tumors or epithelial tissues, especially *in vivo*, might ultimately prove valuable for prostate cancer immunotherapy. Our studies explore a new paradigm in which we will exploit blockade of T cell purigenic receptors A2a and A2b (using caffeine) to alleviate tumor-induced impairments in T cell function to potentiate T cell-mediated immunotherapeutic responses to treat established prostate tumors in mice.

## INTRODUCTION.

In our original application, we proposed three Aims. In Aim 1 we originally proposed to elucidate mechanism(s) whereby AA lowers costimulatory T cell activation thresholds. In Aim 2 we proposed to determine whether AA lowers T cell activation thresholds in men with prostate cancer. In our original Aim 3 we proposed to test whether combination regimens incorporating AA plus chemotherapy can facilitate synergistic responses to immunotherapy to improve prostate cancer treatment.

In April 2004, we requested and were granted, permission to modify Aims 2 and 3 of our original proposal to eliminate scientific overlap with an RO1 award that we received in 2004. Hence, in a revised SOW, we proposed to exploit caffeine-mediated blockade of T cell purigenic receptor in combination with CTLA-4 blockade in order to enhance T cell responses to treat advanced prostate tumors in mice. The rationale for these experiments emanated from recent studies reported by Sitkovsky et al suggesting that full-blown anti-tumoral T cell responses may be severely inhibited by high intratumoral concentrations of adenosine, and that blockade of T cell purigenic receptors A2a and A2b may alleviate tumor-induced impairments of T cell function.<sup>1,2</sup> Specifically, it had long been recognized that "established" tumors produced relatively high concentrations of intratumoral adenosine that emanate from shifts in oxidative to anaerobic glycolytic respiration within the hypoxic intratumoral microenvironment. Consequently, large and relatively hypoxic tumors were anticipated to produce higher levels of adenosine. Dr. Sitkovsky's group had also published that naïve T cells constitutively expressed A2a adenosine receptor and activated T cells further expressed both A2a as well as A2b adenosine receptors. Blockade of T cell adenosine receptors by a relatively non-specific purigenic antagonist, caffeine, was shown to markedly potentiate T cell responses both *in vitro* and *in vivo*. Finally, it has been demonstrated that T cells from A2 receptor-deficient transgenic mice could mediate complete rejection of established tumors following their adoptive transferred into the tumor-bearing host. Collectively, these findings strongly suggested that adenosine produced by established tumors might inhibit T cell responses by binding to T cell purigenic receptors which, in turn, downregulate T cell-mediated antitumoral immunity.

Prompted by these observations, we proposed to investigate whether T cell purigenic receptor blockade might enhance the effectiveness of various forms of immunotherapy to treat very established tumors in our prostate cancer murine models. Specifically, in our new Aim 2, we proposed to elucidate mechanism(s) whereby adenosine inhibits costimulatory T cell activation and the mechanism(s) whereby caffeine-mediated purigenic receptor blockade promotes costimulatory T cell activation. In our new Aim 3 we proposed to test whether *in vivo* caffeine-mediated blockade of T

cell purigenic receptor plus immunotherapy can act synergistically to improve prostate cancer treatment.

## BODY.

To date, we have completed the objectives outlined in our original Aim 1. This information has now been published in the Journal of Immunology 173:6098-6108, 2004. Additionally, we have data to address Aims 2 & 3 in our revised SOW. The findings of our most recent studies are summarized in detail in the following paragraphs.

Figure 1

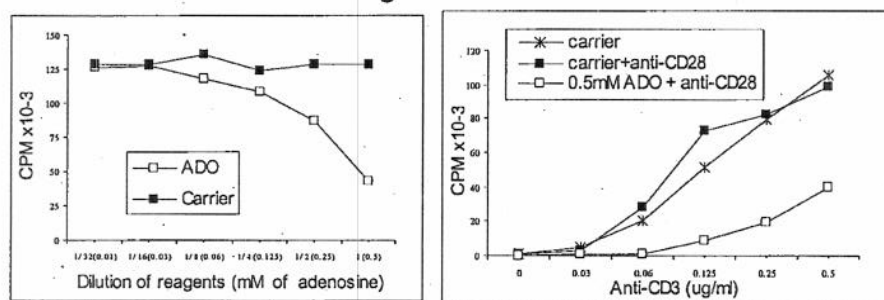


Figure 1. Adenosine (ADO) inhibits the proliferation of T cells in the presence of costimulatory signals.  $2 \times 10^6$  cells/well of purified T cells were cultured for 72 hours with pre-coated graded anti-CD3 with or without soluble anti-CD28 ( $2 \mu\text{g/ml}$ ) in the presence of control carrier (diluted DMSO) or graded concentrations of adenosine. The proliferation of T cells was quantified by the incorporation of  $^3\text{H}$ -thymidine which was pulsed 18 h before harvesting the cells.

Between mid 2004 until mid 2005, we conducted a novel series of studies to test whether caffeine-mediated T cell purigenic receptor blockade could facilitate immunotherapeutically-induced T cell-mediated responses directed against established ectopic and autochthonous prostate tumors in the transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model. Our initial *in vitro* experiments in Figure 1 (above) confirmed that adenosine was capable of inhibiting activated T cell proliferation in response to anti-CD3 (shown) or anti-CD3/anti-CD28 costimulation. Hence to address our Aim 2, we explored several mechanism(s) whereby caffeine-mediated purigenic receptor blockade might promote costimulatory T cell activation. These *in vitro* experiments were conducted to test whether caffeine-mediated blockade of T cell purigenic receptor might promote T cell expansion by pre-empting T cell apoptosis (by TUNEL). In repeated experiments using bead-purified T cells from normal donor mice, and in contrast to what we observed in our initial studies, we observed no changes in rates of T cell apoptosis during anti-CD3 stimulation or anti-CD3/anti-CD28 costimulation in the presence or absence of caffeine. Perform *in vitro* T cell costimulation CD3/CD28 assays (as in our original proposal) in the presence or absence of adenosine and/or caffeine. On the other hand, we did confirm that T cell proliferation (measured by  $^3\text{H}$ -thymidine incorporation) was facilitated by purigenic receptor blockade (+/- adenosine) in response to anti-CD3 or anti-CD3/anti-CD28 costimulation. Hence, Task 1 of Aim 2 was completed [Task 1: Perform *in vitro* T cell costimulation CD3/CD28 assays in the presence or absence of adenosine and/or caffeine. Measure proliferation by  $^3\text{H}$ -thymidine incorporation as well as T cell apoptosis by TUNEL].

To address Task 2 of Aim 2, we began working out a protocol that would enable us to provide constant and prolonged intervals of *in vivo* purigenic receptor blockade through the use of time-release caffeine pellets placed surgically at subcutaneous sites into mice. We deemed this step to be vital since the original papers by Sitkovsky et al had employed delivery of caffeine to mice via

drinking water or intraperitoneal injection which, in our hands, failed to provide continuous and reliable rates of in vivo purigenic blockade owing to the extremely short half-life of caffeine in mice ( $t_{1/2} = 51$  minutes). Moreover, we observed that most mice tended to refuse to drink water with higher concentrations of caffeine. Thus, we explored alternative approaches to dosing mice with caffeine. In these studies, cohorts of mice were surgically implanted with a range of subcutaneous time-release caffeine pellets (1, 5 & 10 mg; 21, 60 & 90-day release formulations; Innovative Research of America) to identify maximal dosages that would provide long-term purigenic receptor blockade as a lead up to our antitumoral immunotherapy experiments proposed in our new Aim 3 (Test whether in vivo caffeine-mediated blockade of T cell purigenic receptor plus immunotherapy can act synergistically to improve prostate cancer treatment). In these experiments, we observed that mice receiving 5 mg subcutaneous subcutaneous time-release tablets tolerated treatment well. In contrast, mice receiving 10 mg formulations exhibited signs of lethal neurologic and cardiovascular overstimulation. Based on these experiments, we selected the 5 mg caffeine formulations and were poised to pursue our antitumoral studies as outlined in Aim 3 beginning mid 2005.

### **KEY RESEARCH ACCOMPLISHMENTS.**

- Original Aim 1. **Completed**
- Revised Aim 2. Elucidate mechanism(s) whereby adenosine inhibits costimulatory T cell activation and the mechanism(s) whereby caffeine-mediated purigenic receptor blockade promotes costimulatory T cell activation. **Completed.**
- Revised Aim 3. Test whether in vivo caffeine-mediated blockade of T cell purigenic receptor plus immunotherapy can act synergistically to improve prostate cancer treatment. **Studies Initiated.**

### **REPORTABLE OUTCOMES TO DATE.**

1. Roden, M. Moser and E.D. Kwon. "Androgen withdrawal increases lymphocyte levels and enhances susceptibility of T cells to costimulatory activation to boost antigen-specific immunity". In preparation.

### **CONCLUSIONS.**

Taken together, our studies indicate that in vivo blockade of T cell purigenic receptors A2a and A2b promotes T cell proliferation and, thereby, may facilitate T cell-mediated immunity against established tumors that tend to produce high levels of adenosine due to hypoxia and upregulated metabolic activity. Moreover, we have now established a viable method for sustained delivery of caffeine into mice to provide prolonged systemic purigenic receptor blockade. At present, we are poised to execute Aim 3 of our proposed studies in which we will test whether in vivo caffeine-mediated blockade of T cell purigenic receptor plus immunotherapy can act synergistically to improve prostate cancer treatment. The results of these experiments will be summarized in our annual report in 2006. Such findings could have significant implications for therapy once confirmed and then translated into the clinical setting.

### **REFERENCES.**

1. Sitkovsky MV et al. Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2a receptors. *Annu Rev Immunol* 22:657-82, 2004.

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